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RNA Polymerase Bypass at Sites of Dihydrouracil: Implications for Transcriptional Mutagenesis

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Dihydrouracil (DHU) is a major base damage product formed from cytosine following exposure of DNA to ionizing radiation under anoxic conditions. To gain insight into the DNA lesion structural requirements for RNA polymerase arrest or bypass at various DNA damages located on the transcribed strand during elongation, DHU was placed onto promoter-containing DNA templates 20 nucleotides downstream from the transcription start site. In vitro, single-round transcription experiments carried out with SP6 and T7 RNA polymerases revealed that following a brief pause at the DHU site, both enzymes efficiently bypass this lesion with subsequent rapid generation of full-length runoff transcripts. Direct sequence analysis of these transcripts indicated that both RNA polymerases insert primarily adenine opposite to the DHU site, resulting in a G-to-A transition mutation in the lesion bypass product. Such bypass and insertion events at DHU sites (or other types of DNA damages), if they occur in vivo, have a number of important implications for both the repair of such lesions and the DNA damage-induced production of mutant proteins at the level of transcription (transcriptional mutagenesis).

The transcription of genes containing DNA damage has several important potential consequences for a cell. Certain lesions, such as UV light-induced cyclobutane pyrimidine dimers and psoralen adducts, have the ability to permanently arrest RNA polymerase at the site of damage during the elongation stage of transcription (9, 28) and, if left unrepaired, block gene expression, which could result in cell death (10). The arrest of an elongation complex is thought to be an important signal for attracting components of the DNA excision repair machinery whose actions result in removal of the damage, restoration of the DNA to its original undamaged state, and ultimately, restoration of transcription (23–26). A large body of literature indicates that such damages, when located on the template strands of actively transcribed genes, are repaired preferentially compared with the genome overall (1, 11, 12, 17, 18). Although the ability of a particular lesion to block RNA polymerase is of primary importance for preferential repair of that lesion, there are several examples in which some types of frequently occurring, spontaneous DNA damages, such as abasic sites (31) and 8-oxoguanine (3), are efficiently bypassed by the transcription elongation complex and, presumably, not subject to transcription-coupled repair. Abasic sites and 8-oxoguanine are also miscoding lesions for RNA polymerase in vitro, causing a majority of base substitutions in the resulting runoff transcripts. The biological importance of such transcriptional insertion events is unknown.

Pyrimidine ring saturation products are an important type of DNA damage and are introduced into cellular DNA by a wide variety of physical and chemical agents (20). 5,6-Dihydrouracil (DHU) is a good model of base damage for studying the potential biological effects of pyrimidine ring saturation products for several reasons (Fig. 1A). It is formed in substantial amounts in DNA exposed to ionizing radiation under anoxic conditions, and it is efficiently removed from DNA by *Escherichia coli* endonuclease III, a base excision repair enzyme that recognizes a wide variety of pyrimidine base damage products (7). DHU, unlike most other pyrimidine ring saturation, ring cleavage, and ring contraction products, is relatively stable under the conditions of solid-phase oligonucleotide synthesis (22) and thus can be directly incorporated into virtually any DNA sequence for a variety of purposes. The structure of DHU suggests that it is not likely to cause major structural distortions when present in duplex DNA (5). No information exists concerning the potential toxicity or mutagenicity of DHU, and its effects on the DNA replication and transcription machinery are unknown.

Previous investigations of the effects of various DNA damages on the transcription elongation process in vitro have focused primarily on relatively bulky, distortive lesions which permanently arrest the progression of RNA polymerase (4, 9, 28). In contrast, only a few studies have examined in detail the interaction of RNA polymerase and small, nondistortive DNA lesions. In this regard, previous work from our laboratory has centered on noninformational DNA damages, such as abasic sites (31) and DNA strand breaks (32), which are incapable of directing base pairing. One previous study has demonstrated that T7 RNA polymerase can bypass 8-oxoguanine (a DNA lesion capable of base pairing) when the lesion is located on the template strand (3). However, because the conditions of the in vitro transcription reactions used in that study allowed each 8-oxoguanine-containing template to be utilized multiple times by the RNA polymerase during an extended period of time (15 to 60 min), it was not possible to obtain information concerning the kinetics of the lesion bypass process (e.g., the degree of stalling that might have taken place) and, hence, the relative efficiency with which the damaged template is transcribed.

We wished to determine the behavior of RNA polymerase at a nondistortive base damage site, DHU, that was capable of base pairing and to utilize transcription conditions under which

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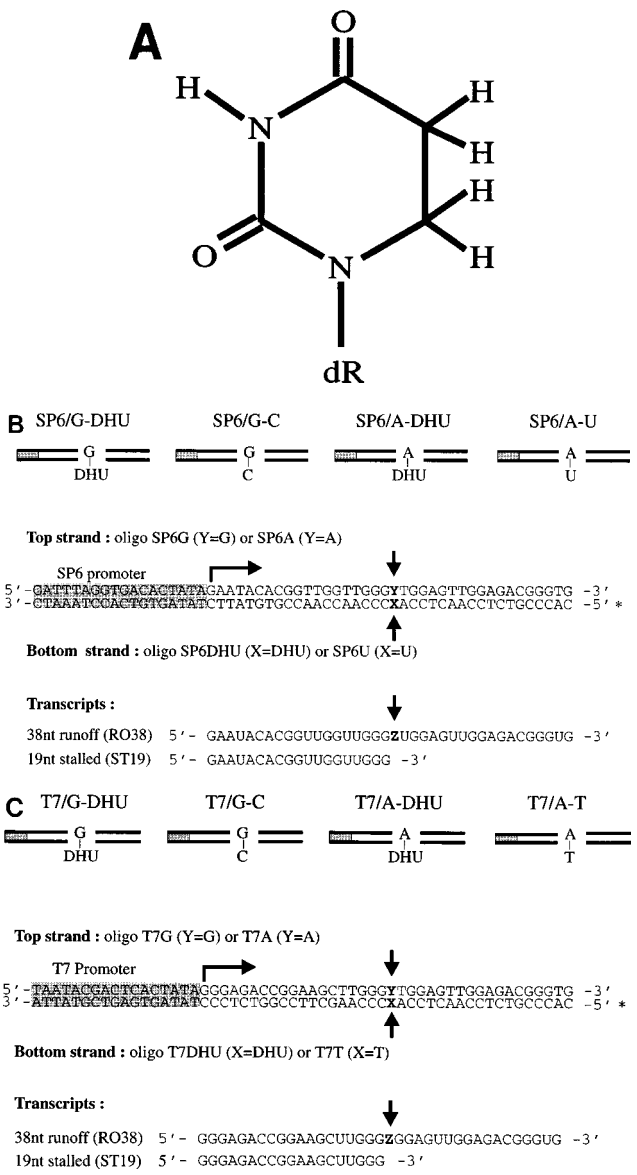

FIG. 1. (A) Structure of DHU. DHU is generated from cytosine by deamination and addition of two hydrogen atoms at the C-5 and C-6 positions. dR, deoxyribose. (B) SP6 RNA polymerase transcription templates (upper schematics) SP6/G-DHU, SP6/G-C, SP6/A-DHU, and SP6/A-U contain DHU, cytosine, DHU, and uracil on the template strand at position X (vertical arrow, bottom strand), respectively. The asterisk indicates the 5′-end-labeled terminus of the DNA template strand. The shaded blocks indicate the SP6 RNA polymerase promoter. The horizontal arrow indicates the transcription start site and direction of transcription. The nucleotide at position Y (vertical arrow, top strand) is 20 nucleotides downstream from the transcription start site on the nontemplate strand and corresponds to guanine in oligonucleotide SP6G and adenine in SP6A (upper schematics). Predicted full-length, runoff (RO38), and stalled (ST19) transcripts generated from these templates are shown. Position Z (downward vertical arrow) in RO38 represents the base inserted opposite to position X on the template strand. ST19 is generated when SP6 RNA polymerase is stalled at position X. (C) T7 RNA polymerase transcription templates T7/G-DHU, T7/G-C, T7/A-DHU, and T7/A-T contain DHU, cytosine, DHU, and thymine on the template strand at position X, respectively (upper schematics). The asterisk indicates the 5′-end-labeled terminus of the DNA template strands, and the shaded blocks indicate the T7 RNA polymerase promoter. Other designations and transcripts produced by T7 RNA polymerase are similar to those described for panel B. nt, nucleotide.

only a single round of elongation was allowed. In this way, the temporal relationship between RNA polymerase and events taking place at the site of damage (arrest, stalling, or bypass) can be directly determined. In addition, if bypass of the DNA lesion was observed, we wished to identify the nature of the base(s) inserted opposite to the DHU site to determine if transcriptional miscoding occurs. In this investigation, we constructed transcription templates containing DHU at a single location on the template strand downstream from the start of transcription. We compared the behavior of two RNA polymerases (T7 and SP6) when encountering DHU and determined the nature of the full-length transcripts resulting from bypass of this lesion. The results of these studies indicate that following brief stalling at DHU, RNA polymerase efficiently bypasses this lesion and generates full-length transcripts containing primarily mutagenic insertions at the site of damage. These findings are discussed with respect to the implications for defining the spectrum of DNA damages that may be subject to transcription-coupled repair, as well as for the concept of transcriptional mutagenesis.

MATERIALS AND METHODS

Materials. *E. coli* endonuclease III was a gift from Richard P. Cunningham (Albany, N.Y.). DHU was purchased from Sigma. The DHU dimethoxytrityl-blocked phosphoramidite building block was synthesized by Glenn Research (Sterling, Va.). Oligonucleotides were synthesized by the Emory University Microchemical Facility and purified by polyacrylamide gel electrophoresis (15). The sequence of each oligonucleotide was verified by Maxam and Gilbert chemical DNA sequencing (16). T7 and SP6 RNA polymerases were purchased from Promega and Stratagene, respectively. Heparin and RNase inhibitor were purchased from Sigma. T4 polynucleotide kinase was purchased from New England Biolabs. Calf intestinal phosphatase was from Boehringer Mannheim. RNA sequencing kits (nuclease method) were from United States Biochemicals. [α-^{32}P]CTP (specific activity, 3,000 Ci/mmol) and [γ-^{32}P]ATP (3,000 Ci/mmol) were from Amersham. High-pressure liquid chromatography-purified nucleoside triphosphates were purchased from Pharmacia.

Construction of DNA templates. To construct DHU-containing DNA template SP6/G-DHU, oligonucleotide SP6G (Fig. 1B) was 5′ end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (19). A 100 μM concentration of 5′-end-labeled oligonucleotide SP6G was mixed with 100 μM oligonucleotide SP6DHU (Fig. 1B) in 10 mM $MgCl_2$. The mixture was heated to 70°C for 10 min and cooled to room temperature for 4 h. Annealed DNA template was purified from a 20% nondenaturing polyacrylamide gel as previously described (30). DNA templates SP6/G-C, SP6/A-U, SP6/A-DHU, T7/A-T, T7/A-DHU, T7/G-C, and T7/G-DHU (Fig. 1B and C) were constructed by a similar approach.

Single- and multiple-round transcription experiments. Single-round transcription experiments were carried out by preincubation of 5 pmol of DNA template with 10 mM dithiothreitol–20 mM Tris-HCl (pH 7.9)–3 mM $MgCl_2$–5 mM NaCl–50 μM ATP, UTP, and GTP–20 U of SP6 (1.2 pmol) or T7 (0.6 pmol). RNA polymerase–18 U of RNase inhibitor for 8 min at room temperature. After that, heparin (250 μg/ml), CTP (10 μM), and 10 μCi of [α-^{32}P]CTP (3,000 Ci/mmol) were added to the preincubation mixture. Two-microliter aliquots were removed, and reactions were terminated with stop loading buffer (9.8 M urea, 50 mM EDTA, 0.1% xylene cyanol) at different time points. The ^{32}P-labeled transcripts were analyzed on a 15% denaturing polyacrylamide gel and subjected to autoradiography.

Multiple-round transcription experiments were carried out as previously described (31), with 1 pmol of DNA template in 40 mM Tris-HCl (pH 7.9)–6 mM $MgCl_2$–10 mM NaCl–10 mM dithiothreitol–0.5 mM ATP–0.5 mM GTP–0.5 mM UTP–10 μM CTP–10 μCi of [α-^{32}P]CTP–18 U of RNase inhibitor–20 U of SP6 or T7 RNA polymerase. RNA molecular size marker ladders were generated by alkaline hydrolysis of multiple-round transcription products, as previously described (31), with 50 mM Na_3PO_4, pH 12, for 20 min at 70°C.

RNA sequencing. RNA transcripts for sequence analysis were generated as described above from multiple-round transcription experiments, except that 0.5 mM CTP was used in place of 10 μM CTP and 10 μCi of [α-^{32}P]CTP. Unlabeled transcripts were treated with calf intestinal phosphatase to remove the 5′-terminal phosphate. After gel purification, RNA transcripts were 5′ end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (15). RNA sequencing reactions were carried out with base-specific RNases as described in the RNA Sequencing Kit (United States Biochemicals).

Quantitation of bypass efficiency of SP6 and T7 RNA polymerases. Three separate single-round transcription experiments for each template were carried out, and the relative amounts of stalled (ST19) and full-length (RO38) RNA transcripts at each time point were quantitated by PhosphorImager (Molecular Dynamics 445 SI) analysis of the polyacrylamide gel. For templates transcribed

by SP6 RNA polymerase, there are three cytosine residues in each full-length transcript (RO38) and two cytosine residues in each stalled transcript (ST19). The percentage of ST19 was calculated as {[ST19 × (3/2)]/[ST19 × (3/2) + RO38]} × 100, and the percentage of RO38 was calculated as {RO38/[ST19 × (3/2) + RO38]} × 100 (32). For templates transcribed by T7 RNA polymerase, there are four cytosine residues in each full-length transcript and three cytosine residues in each stalled transcript. The percentage of ST19 was calculated as {[ST19 × (4/3)]/[ST19 × (4/3) + RO38]} × 100, and the percentage of RO38 was calculated as {RO38/[ST19 × (4/3) + RO38]} × 100.

RESULTS

Generation of DNA templates containing a single DHU lesion for in vitro transcription. Duplex DNA templates SP6/G-DHU, SP6/A-DHU, T7/G-DHU, and T7/A-DHU were constructed by chemical synthesis of a T7 or SP6 RNA polymerase template strand containing DHU at nucleotide position 19 (from the 5′ end), followed by annealing to a complementary strand (Fig. 1B and C). The resulting duplex templates contained either the T7 or SP6 promoter and a transcribable region (38 nucleotides long) containing DHU 20 nucleotides downstream from the start of transcription. Similar constructs containing cytosine (templates SP6/G-C and T7/G-C), thymine (template T7/A-T), or uracil (template SP6/A-U) at the same location were also generated. This collection of templates allows direct comparison of the behavior of two different RNA polymerases at a DNA lesion site placed within the same flanking sequence context and located opposite to either G or A on the nontemplate strand. Transcription of the undamaged templates (SP6/G-C, T7/G-C, and T7/A-T) or the uracil-containing template (SP6/A-U) should generate full-length runoff transcripts 38 nucleotides long (RO38). RNA polymerase arrest or stalling at the DHU site (templates SP6/G-DHU, SP6/A-DHU, T7/G-DHU, and T7/A-DHU) should generate transcripts 19 nucleotides long (ST19).

The constructs were 5′ end labeled on the template strand to facilitate subsequent template analysis and to allow precise control of the amounts of DNA used in various transcription experiments on the basis of the known specific activities of the end-labeled templates (31). The placement of DHU at nucleotide position 19 was verified by treatment of templates SP6/G-DHU, SP6/A-DHU, T7/G-DHU, and T7/A-DHU with *E. coli* endonuclease III, which causes DNA strand scission at sites of DHU via a combined *N*-glycosylase–AP lyase activity (8), generating an 18-nucleotide DNA cleavage product (Fig. 2).

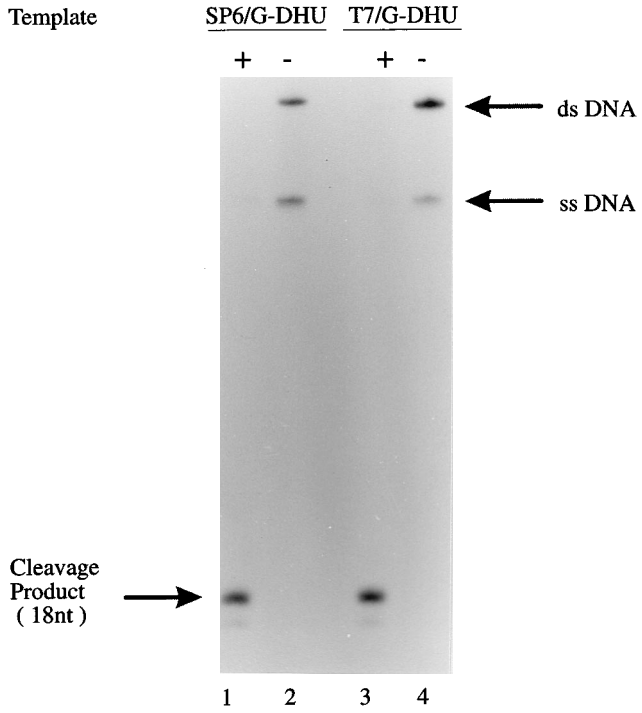

FIG. 2. DNA template analysis. Templates SP6/G-DHU (lanes 1 and 2) and T7/G-DHU (lanes 3 and 4) were 5′ end labeled on the transcribed strand and treated with *E. coli* endonuclease III (lanes 1 and 3) or left untreated (lanes 2 and 4) and then subjected to electrophoresis on a 15% denaturing polyacrylamide gel and autoradiography as described in Materials and Methods. The arrows indicate the positions of undenatured duplex DNA (dsDNA), the 5′-end-labeled, single-stranded transcribed strand (ssDNA), and the 18-nucleotide (nt) DNA cleavage product resulting from endonuclease III cleavage of the transcribed strand at DHU sites.

SP6 RNA polymerase behavior at DHU sites. To determine the interaction of SP6 RNA polymerase with DHU during elongation, comparative single-round transcription experiments were carried out with templates SP6/G-C, SP6/G-DHU, SP6/A-DHU, and SP6/A-U. Each template molecule is utilized only once by a single molecule of RNA polymerase, and the transcription products generated reflect a single, promoter-dependent elongation event (31). Single-round transcription experiments with undamaged control template SP6/G-C (containing cytosine on the template strand 20 nucleotides downstream from the start of transcription) produced full-length runoff transcripts 38 nucleotides long (RO38), as expected (Fig. 3A, lanes 10 to 17). Under these conditions, elongation was essentially complete after 60 s and was followed by the appearance of species 39 and 40 nucleotides long which are the result of nontemplated additions to the 3′ end of RO38; this is a property of several of RNA polymerases, including SP6 RNA polymerase (13, 31). Transcription of template SP6/G-DHU (containing DHU in place of cytosine on the template strand 20 nucleotides downstream from the start of transcription) resulted in the initial production of stalled transcripts 19 nucleotides long (ST19) which rapidly disappeared at later times and were converted into full-length transcripts (Fig. 3A, lanes 2 to 9). Quantitation of the relative amounts of ST19 and RO38 indicated that stalling of SP6 RNA polymerase at the DHU site reaches a maximum at 5 s following the initiation of elongation, followed by rapid bypass and chain extension into full-length transcripts (Fig. 4A). The generation of RO38 from template SP6/G-DHU also indicates that a base is inserted opposite to DHU during transcription elongation.

We wished to investigate whether or not the nature of the base located on the nontemplate strand opposite to the DHU site had any effect on RNA polymerase stalling or bypass of this lesion. Single-round transcription experiments were carried out with template SP6/A-DHU, which is identical to SP6/G-DHU except that guanine was replaced by adenine as the base opposite to DHU (Fig. 1B). The behavior of SP6 RNA polymerase at the DHU site on template SP6/A-DHU was nearly identical to that observed for SP6/G-DHU with respect to both the nature of the transcripts generated (Fig. 3B, lanes 2 to 9) and the kinetics of lesion bypass (Fig. 4B). As an additional control, we utilized template SP6/A-U, containing uracil (a spontaneous deamination product of cytosine [14, 27]) in place of DHU, and observed no stalling at the position of uracil with the rapid generation of full-length transcripts (RO38) similar to that obtained with undamaged template SP6/G-C (Fig. 3B, lanes 10 to 17). Inclusion of template SP6/A-U allowed direct comparison of the effects of pyrimidine ring saturation between otherwise identical structures (uracil and DHU) on the behavior of SP6 RNA polymerase. We concluded that uracil ring saturation causes temporary poly-

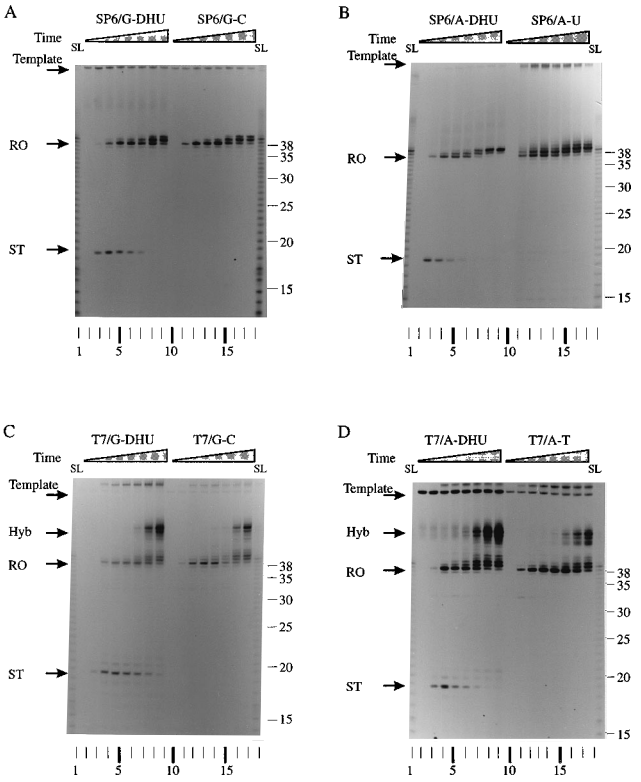

FIG. 3. Single-round transcription experiments with DHU-containing and undamaged templates. In A through D, ST indicates stalled transcripts (19 nucleotides) and RO indicates full-length runoff transcripts (38 nucleotides). Lanes 2 to 9 and 10 to 17 correspond to transcription products generated at 5 s, 12 s, 20 s, 30 s, 1 min, 2 min, and 4 min following the start of elongation, respectively. RNA molecular size marker ladders (SL) are in lanes 1 and 18 of each gel, and lengths are indicated alongside lane 18 (in nucleotides). The arrow at top of each gel indicates the position of the 5′-end-labeled DNA template. (A) Transcription experiments with SP6 RNA polymerase and templates SP6/G-DHU (lanes 2 to 9) and SP6/G-C (lanes 10 to 17). (B) Transcription experiments with SP6 RNA polymerase and templates SP6/A-DHU (lanes 2 to 9) and SP6/A-U (lanes 10 to 17). (C) Transcription experiments with T7 RNA polymerase and templates T7/G-DHU (lanes 2 to 9) and T7/G-C (lanes 10 to 17). Hyb indicates a DNA-RNA hybrid species. (D) Transcription experiments with T7 RNA polymerase and templates T7/A-DHU (lanes 2 to 9) and T7/A-T (lanes 10 to 17). Hyb indicates a DNA-RNA hybrid species.

merase stalling at the site of damage. We also concluded from these results that the nature of the base on the nontemplate strand located opposite to the lesion site has no effect on the interaction between the SP6 RNA polymerase active site and DHU.

T7 RNA polymerase behavior of DHU sites. We wished to determine whether other RNA polymerases exhibited stalling-bypass behavior at DHU sites on the template strand. Single-round transcription experiments with T7 RNA polymerase were carried out with templates T7/G-DHU, T7/G-C, T7/A-DHU, and T7/A-T. Transcription of undamaged control templates T7/G-C and T7/A-T generated full-length runoff transcript RO38, as expected (Fig. 3C and D, lanes 10 to 17), which was extended at later times by two additional nucleotides via nontemplated additions (13, 32). The results of T7 RNA polymerase single-round transcription of DHU-containing templates T7/G-DHU and T7/A-DHU (Fig. 3C and D, lanes 2 to 9) were remarkably similar to those obtained with SP6 RNA polymerase with templates SP6/G-DHU and SP6/A-DHU. For both polymerases, the kinetics of stalling at DHU and chain extension of ST19 into RO38 were nearly identical (Fig. 4). Furthermore, as in the case of SP6 RNA polymerase, changing the base on the nontemplate strand opposite to DHU had no effect on the behavior of T7 RNA polymerase. In the T7 RNA polymerase experiments, an additional, slowly migrating species (Hyb) was observed at later times during elongation (Fig. 3C and D). This species was found to be a noncovalent hybrid of the DNA template and the RNA transcript on the basis of its lability following DNase I, RNase H, and alkali treatments (unpublished results; 30, 33). Thus, at later times during elongation, the amount of full-length transcript (RO38 and its nontemplated additions) measured is an underestimate due to the association of a fraction of RO38 with the end-labeled DNA template. Taken together, the results of the SP6 and T7 RNA polymerase transcription experiments indicate that polymerase temporarily stalls at DHU sites and that the stalled ternary complexes are still transcriptionally engaged with subsequent base insertion opposite to the DHU and further chain elongation into full-length transcripts.

Sequence analysis of transcripts. The precursor base for DHU formation is cytosine (7). Therefore, if any base other than guanine is inserted opposite to DHU by RNA polymerase, the resulting transcript will contain a base substitution mutation. To determine the nature of the base that was inserted opposite to DHU at nucleotide position 20 on RO38 by SP6 and T7 RNA polymerases, full-length runoff transcripts (RO38) were generated under multiple-round transcription conditions from each of the eight templates described above and were subjected to direct RNA sequence analysis with base-specific RNases (see Materials and Methods). As expected, adenine was inserted opposite to uracil on template SP6/A-U

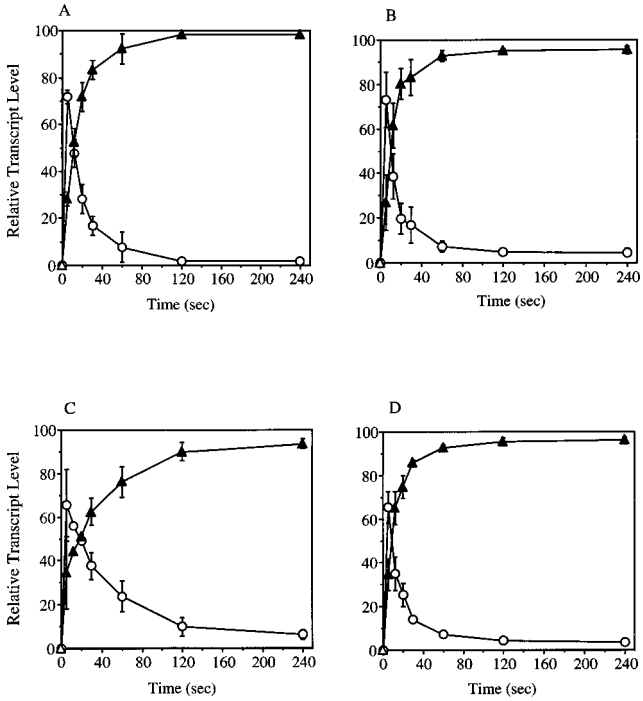

FIG. 4. Bypass efficiency of RNA polymerase at DHU sites on the transcribed strand. The relative amounts of stalled transcript ST19 (○) and full-length transcript RO38 (▲) were calculated as described in Materials and Methods. (A and B) Bypass efficiency of SP6 RNA polymerase at DHU in templates SP6/G-DHU and SP6/A-DHU, respectively. (C and D) Bypass efficiency of T7 RNA polymerase at DHU in templates T7/G-DHU and T7/A-DHU, respectively. Three separate single-round transcription experiments were conducted for each template. Error bars represent standard deviations.

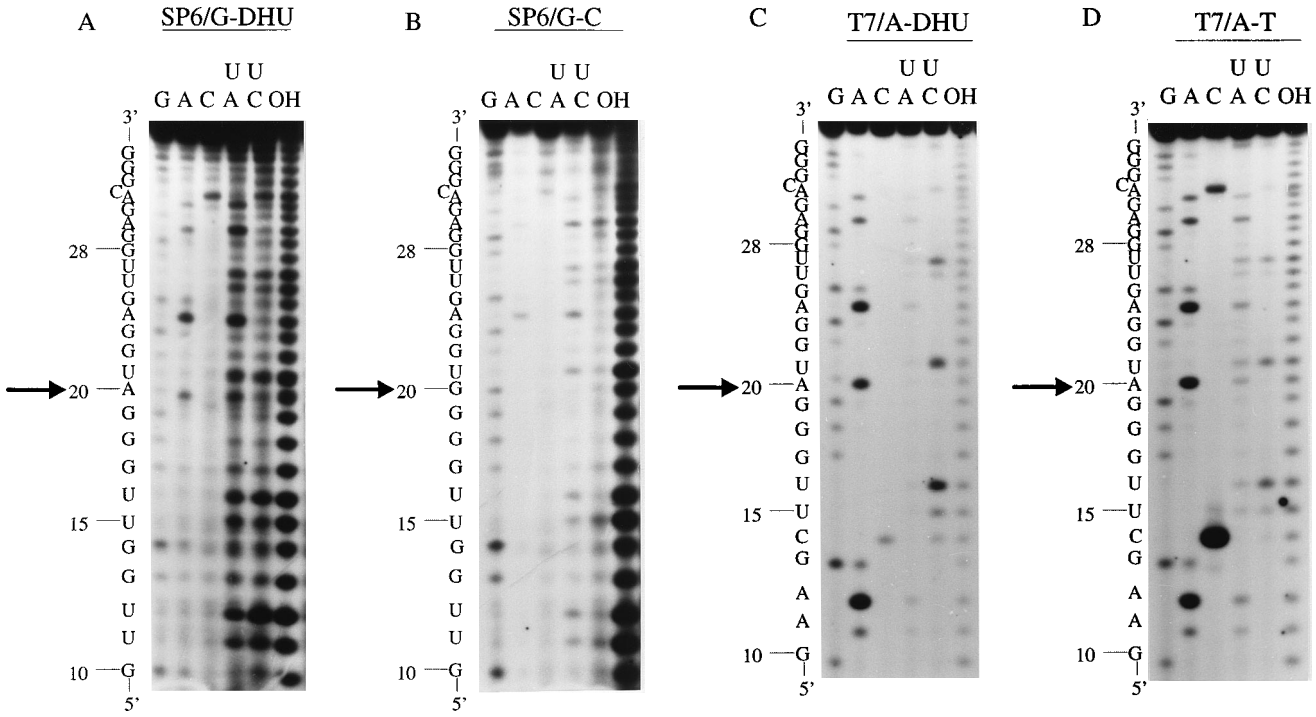

FIG. 5. Transcript sequence analysis. SP6 and T7 RNA polymerase-generated transcripts were produced under multiple-round transcription conditions and 5′ end labeled as described in Materials and Methods. RNA sequencing was carried out with base-specific RNases (lanes G, A, C, UA, and UC) as described in Materials and Methods. Each arrow indicates the transcript base inserted (position Z) opposite to position X on the transcribed strand (Fig. 1B and C). The RNA size ladder was generated by alkaline hydrolysis (lanes OH) of the 5′-end-labeled, full-length runoff transcripts. RO38 sequences were generated by SP6 RNA polymerase transcription of templates SP6/G-DHU (A), SP6/G-C (B), T7/A-DHU (C), and T7/A-T (D).

by SP6 RNA polymerase (data not shown). Sequence analysis of the transcripts produced from undamaged control templates SP6/G-C, T7/A-T (Fig. 5B and D), and T7/G-C (data not shown) by RNA polymerase revealed the expected nucleotide sequence complementary to the template strand. The sequences obtained from the full-length transcripts (RO38) produced from DHU-containing templates SP6/G-DHU and T7/A-DHU indicated that both SP6 and T7 RNA polymerases insert primarily adenine opposite to the DHU site (Fig. 5A and C). In addition, a low level of guanine insertion opposite to DHU was also observed. Similar results were obtained for the transcripts produced from the other two DHU-containing templates, SP6/A-DHU and T7/G-DHU (data not shown). The transcript sequence analysis results are summarized in Table 1.

TABLE 1. Bases inserted at nucleotide position 20[a] on RO38[b]

RNA polymerase	Template base	Nontemplate base	Transcription template	Base inserted
SP6	DHU	G	SP6/G-DHU	A>>G
SP6	DHU	A	SP6/A-DHU	A>>G
SP6	C	G	SP6/G-C	G
SP6	U	A	SP6/A-U	A
T7	DHU	G	T7/G-DHU	A>>G
T7	DHU	A	T7/A-DHU	A>>G
T7	C	G	T7/G-C	G
T7	T	A	T7/A-T	A

[a] Corresponds to position Z (Fig. 1B and C) located 20 nucleotides downstream from the 5′ end of RO38.

[b] Full-length runoff transcripts (RO38) produced by SP6 or T7 RNA polymerase transcription of the templates indicated were subjected to direct RNA sequence analysis as described in Materials and Methods.

DHU causes primarily G-to-A transition mutations in the resulting transcripts, and no other types of mutations (e.g., deletions or insertions) were detected. Thus, DHU is mutagenic at the level of transcription.

DISCUSSION

DHU, when located on the template strand of a transcribed segment of DNA, is efficiently bypassed by both SP6 and T7 RNA polymerases. Unlike cyclobutane pyrimidine dimers, which cause permanent arrest of RNA polymerase at the damage site until they are removed by the DNA excision repair machinery (24), DHU elicits only brief polymerase stalling, followed by rapid chain extension into full-length transcripts. Transcriptional bypass of DHU is accompanied by insertion of adenine opposite to this damage, and such an event is equivalent to a base substitution mutation at the level of RNA synthesis. One possibility is that the preference for adenine insertion is due to the predicted Watson-Crick base pair that can form between DHU and adenine, which is similar to a uracil-adenine base pair (21). The less frequent insertion of guanine can also be understood in terms of its predicted ability to form a wobble base pair with DHU (5). The brief period of stalling observed at the DHU site could be attributed to the increased time required at the RNA polymerase active site for either adenine or guanine base pairing to occur with DHU, whose hydrogen bond donor (H3) and acceptor (O4) atoms are no longer coplanar because of the saturation of the DHU pyrimidine ring.

The bypass of DHU by RNA polymerase places DHU into a group of DNA damages which includes abasic sites (31), 8-oxoguanine (3), and certain types of DNA single-strand

TABLE 2. Predicted mutagenic effects of replacing guanine with adenine in *E. coli* mRNA

DHU precursor base on template strand	RNA base sequence change in codon	Mutation type[a]	No. of possible different mutations[b]	Occurrence (%)[c]
C	G→A	Silent	15	36.0
C	G→A	Missense	31	63.0
C	G→A	Nonsense	2	1.0

[a] Type of substitution mutation in mRNA resulting from adenine insertion by RNA polymerase opposite to DHU (originating from cytosine) on the transcribed strand.

[b] Different mutations resulting from replacement of cytosine with adenine in the first, second, or third nucleotide position of a codon.

[c] Frequency of occurrence of a particular mutation type, taking into account the number of possible different mutations and the frequency of codon usage in *E. coli* (29). Value normalized to 100% for codons containing G.

breaks (32). Such DNA damages are all efficiently bypassed by various RNA polymerases, with resulting mutagenic base insertions or deletions as the predominant event occurring opposite to the lesion. These bypass-insertion observations have several important implications for DNA repair, as well as the genetic mechanisms leading to the generation of mutant proteins in cells. Efficient RNA polymerase bypass of DHU, abasic sites, 8-oxoguanine, and strand breaks leads to the prediction that these lesions are not subject to the transcription-coupled repair system that preferentially removes RNA polymerase-arresting lesions such as cyclobutane pyrimidine dimers and psoralen monoadducts from the template strands of actively transcribed genes compared with the overall genome (17–19). A key feature in the transcription-coupled repair mechanism is the ability of the lesion to permanently arrest RNA polymerase at or near the damage site. It is likely that brief stalling by RNA polymerase, followed by efficient bypass, is not sufficient for triggering of a transcription-coupled repair response. Transcribed genes which contain DNA damages that do not permanently arrest RNA polymerase may actually reduce the ability of the cell to repair such lesions by preventing access to DNA repair proteins because of RNA polymerase occupancy of that gene. Such a situation might lead to an increase in mutations arising from unrepaired damage on the transcribed strand of a gene during a subsequent DNA replication cycle and may explain the recent finding that transcriptionally active DNA and enhanced spontaneous mutation rates are associated in *Saccharomyces cerevisiae* (6).

Efficient RNA polymerase bypass of DHU or other damages, if it occurs in vivo, could lead to the generation of mutant proteins via a second route involving a transcriptional miscoding mechanism (transcriptional mutagenesis). Since cytosine is the precursor base for DHU, the predominant change in the transcript mRNA sequence will be a G-to-A transition mutation. In *E. coli*, for example, if codon usage is taken into account (29) and if there is an equal probability that any cytosine in the template strand of a transcribed gene could serve as the precursor for DHU, then the most frequently occurring type of codon change predicted following an adenine insertion opposite to DHU would be a missense mutation leading to an amino acid sequence change in the translated protein (Table 2). Such transcriptional behavior leading to the generation of mutant proteins could have a number of important biological outcomes, especially in a population of nondividing cells. Such mutant proteins could exert toxic effects leading to impaired function or death or initiate events leading to the cell's entry into a replicative cycle and cell division. It has recently been postulated that the phenomenon of directed mutation in *E. coli* may involve the latter possibility, in which transcriptional miscoding at spontaneous DNA base damage results in a transient phenotype, allowing a round of DNA replication to occur, which then permanently fixes the mutation at the unrepaired damage site by a DNA polymerase miscoding event (2). The potential biological endpoints resulting from transcriptional mutagenesis are unknown; this underscores the need for investigation of its occurrence in cells.

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